

## MCK-10, A NOVEL RECEPTOR TYROSINE KINASE

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MCK-10, A NOVEL RECEPTOR TYROSINE KINASE

INSAI *invented by*

1. INTRODUCTION

The present invention relates to the novel family  
5 of receptor tyrosine kinases, herein referred to as  
MCK-10, to nucleotide sequences and expression vectors  
encoding MCK-10, and to methods of inhibiting MCK-10  
activity. The invention relates to differentially  
spliced isoforms of MCK-10 and to other members of the  
10 MCK-10 receptor tyrosine kinase family. Genetically  
engineered host cells that express MCK-10 may be used  
to evaluate and screen drugs involved in MCK-10  
activation and regulation. The invention relates to  
the use of such drugs, in the treatment of disorders,  
15 including cancer, by modulating the activity of  
MCK-10.

2. BACKGROUND

Receptor tyrosine kinases comprise a large family.  
20 of transmembrane receptors which are comprised of an  
extracellular ligand-binding domain and an  
intracellular tyrosine-kinase domain responsible for  
mediating receptor activity. The receptor tyrosine  
kinases are involved in a variety of normal cellular  
25 responses which include proliferation, alterations in  
gene expression, and changes in cell shape.

The binding of ligand to its cognate receptor  
induces the formation of receptor dimers leading to  
activation of receptor kinase activity. The  
30 activation of kinase activity results in  
phosphorylation of multiple cellular substrates  
involved in the cascade of events leading to cellular  
responses such as cell proliferation.

Genetic alterations in growth factor mediated  
35 signalling pathways have been linked to a number of

different diseases, including human cancer. For example, the normal homologs of many oncogenes have been found to encode growth factors or growth factor receptors. This is illustrated by the discovery that the B chain of human PDGF is homologous to the transforming protein of simian sarcoma virus (SSV), the EGF (epidermal growth factor) receptor to *erb B*; the CSF (colony stimulating factor) receptor to *fms*; and the NGF (nerve growth factor) receptor to *trk*. In addition, growth factor receptors are often found amplified and/or overexpressed in cancer cells as exemplified by the observation that the EGF receptor is often found amplified or overexpressed in squamous cell carcinomas and glioblastomas. Similarly, amplification and overexpression of the *met* gene, encoding the HGF receptor, has been detected in stomach carcinomas.

Recently, a number of cDNAs have been identified that encode receptor tyrosine kinases. One such clone, referred to as DDR (discoidin domain receptor), was isolated from a breast carcinoma cDNA library (Johnson et al., 1993, Proc. Natl. Acad. Sci. USA, 90, 5677-57681) and is homologous to MCK-10. In addition, a mouse homologue of MCK-10 has recently been cloned and characterized (Yerlin, M. et al., 1993, Oncogene, 8:2731-2739).

The discovery of novel receptor tyrosine kinase receptors, whose expression is associated with proliferative diseases such as cancer, will provide opportunities for development of novel diagnostic reagents. In addition, the identification of aberrantly expressed receptor tyrosine kinases will lead to the development of therapeutic applications designed to inhibit the activity of that receptor, which may be useful for treatment of proliferative diseases such as cancer.

### 3. SUMMARY OF THE INVENTION

The present invention relates to a novel family of receptor tyrosine kinases, herein referred to as MCK-10 (mammary carcinoma kinase 10), to nucleotide sequences and expression vectors encoding MCK-10, and  
5 to methods of inhibiting MCK-10 activity. The invention is based on the isolation of cDNA clones from a human mammary carcinoma cDNA library encoding the MCK-10 receptor tyrosine kinase.

The invention also relates to differentially  
10 spliced isoforms of MCK-10 and to other members of the MCK-10 family of receptor tyrosine kinases. More specifically, the invention relates to members of the MCK-10 family of receptors tyrosine kinases that are defined, herein, as those receptors demonstrating 80%  
15 homology at the amino acid level in substantial stretches of DNA sequences with MCK-10. In addition, members of the MCK-10 family of tyrosine kinase receptors are defined as those receptors containing an intracellular tyrosine kinase domain and consensus  
20 sequences near the extracellular N-terminus of the protein for the discoidin I like family of proteins. The invention as it relates to the members of the MCK-10 family of receptor tyrosine kinases, is based on the isolation and characterization of a cDNA, herein  
25 referred to as CCK-2, encoding a member of the MCK-10 family of receptor tyrosine kinases.

Northern blot analysis and *in situ* hybridization indicates that MCK-10 is expressed in a wide variety of cancer cell lines and tumor tissue. The MCK-10 or  
30 CCK-2 coding sequence may be used for diagnostic purposes for detection of aberrant expression of these genes. For example the MCK-10 or CCK-2 DNA sequence may be used in hybridization assays of biopsied tissue to diagnose abnormalities in gene expression.

35

The present invention also relates to inhibitors of MCK-10 or CCK-2 receptor activity which may have therapeutic value in the treatment of proliferative diseases such as cancer. Such inhibitors include antibodies to epitopes of recombinantly expressed MCK-10 or CCK-2 receptor that neutralize the activity of the receptor. In another embodiment of the invention, MCK-10 or CCK-2 anti-sense oligonucleotides may be designed to inhibit synthesis of the encoded proteins through inhibition of translation. In addition, random peptide libraries may be screened using recombinantly produced MCK-10 or CCK-2 protein to identify peptides that inhibit the biological activity of the receptor through binding to the ligand binding sites or other functional domains of the MCK-10 or CCK-2 receptor. In a further embodiment of the invention, mutated forms of MCK-10 and CCK-2, having a dominant negative effect, may be expressed in targeted cell populations to inhibit the activity of the endogenously expressed receptors.

#### 4. BRIEF DESCRIPTION OF THE FIGURES

Figures 1A, 1B and 1C. Human MCK-10 nucleotide sequence and deduced amino acid sequence. Regions of interest include the signal sequence (amino acids (aa) 1-18); the Discoidin I-like domain (aa 31-185); the putative precursor cleavage site (aa 304-307); the transmembrane region (aa 417-439); the alternatively spliced sequence I (aa 505-541); the alternatively spliced sequence II (aa 666-671); and the peptide antibody recognition sequences: NT $\alpha$ :aa 25-42, NT $\beta$ :aa 309-321, CT $\beta$ :aa 902-919.

Figure 2. MCK-10 splice variants.

Figures 3A, 3B, 3C and 3D. Human CCK-2 nucleotide sequence and deduced amino acid sequence.

2nd B4  
Figure 4A. Shared sequence homology between MCK-10 and CCK-2.

Figure 4B. Shared regions of homology between MCK-10 and CCK-2.

5 Figure 5A. Northern blot analysis of MCK-10 mRNA in different human tissues. Three micrograms of poly (A)<sup>+</sup> RNA are loaded per lane. The blot is hybridized with a cDNA restriction fragment corresponding to nucleotide 278 to 1983 of MCK-10 (Figures 1A, 1B and 1C) (excluding the 111 bp insertion). As a control, 10 the blot was rehybridized with a glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe (lower panel).

15 Figure 5B. Northern blot analysis of MCK-10 gene in various human breast cancer cell lines. Samples containing three micrograms of poly (A)<sup>+</sup> RNA isolated from different human breast cancer cell lines were analyzed. The position of 28S and 18S ribosomal RNAs is indicated, the lower panel shows the rehybridization with a GAPDH cDNA probe.

20 Figure 5C. Northern blot analysis of MCK-10 mRNA in different human tissues and cell lines of tumor origin. Size markers are indicating 28S and 18S ribosomal RNAs (upper panel). Rehybridization is performed with a GAPDH cDNA probe (lower panel).

25 Figure 6A. Tyrosine phosphorylation of overexpressed MCK-10. The coding cDNAs of MCK-10-1 and MCK-10-2 were cloned into an expression vector and transiently overexpressed in the 293 cell line (human embryonic kidney fibroblasts, ATCC CRL 1573). 30 Portions of cell lysate from either MCK-10-1 or -2 transfected cells or control plasmid transfected cells (mock) were separated on a 7-12% gradient polyacrylamide gel and transferred to nitrocellulose and probed with anti-phosphotyrosine antibodies ( $\alpha$ PY). 35 The incubation of cells with 1mM sodium ortho-vanadate

90 min. prior to lysis is indicated by -/+; (left panel). After removal of the  $\alpha$ PY antibody the blot was reprobbed with an affinity purified polyclonal antiserum raised against the C-terminal octapeptide of MCK-10 ( $\alpha$  MCK-10-C); (right panel). Molecular size markers are indicated in kD.

Figure 6B. Distinct glycosylation of overexpressed MCK-10 splice variants. 293 cells were transfected with MCK-10-1 and -2 as before, metabolically labeled with [ $^{35}$ S]-L-methionine and treated with 10 $\mu$ g/ml tunicamycin overnight as indicated (+), lysed and immunoprecipitated with antisera generated against the N-terminal and C-terminal peptides of MCK-10 ( $\alpha$  MCK-10-N and  $\alpha$  MCK-10-C). The autoradiograph of the SDS-PAGE analysis is shown. Molecular size markers are indicated in kD.

Figure 7. *In situ* hybridization showing specific expression of MCK-10 in epithelial cells of the distal tubuli of the kidney.

Figure 8. *In situ* hybridization showing expression of MCK-10 only in epithelial cells of the distal tubular cells of the kidney.

Figure 9. *In situ* hybridization showing specific expression of MCK-10 in tumor cells of a renal cell carcinoma.

Figure 10. *In situ* hybridization of MCK-10 in the ductal epithelial cells of normal breast tissue.

Figure 11. *In situ* hybridization showing MCK-10 expression in infiltrating tumor cells of a breast carcinoma. The tumor infiltrates the surrounding fat tissue, which is negative for MCK-10 expression.

Figure 12. *In situ* hybridization showing MCK-10 expression in infiltrating tumor cells of a breast carcinoma. The tumor infiltrates the surrounding fat tissue, which is negative for MCK-10 expression.

Figure 13. *In situ* hybridization showing expression of MCK-10 expression in the islet cells of the pancreas.

Figure 14. *In situ* hybridization showing expression of MCK-10 expression in the islet cells of the pancreas.

Figure 15. *In situ* hybridization showing selective expression of MCK-10 in the surface epithelium of the colon in contrast to connective tissue.

Figure 16. *In situ* hybridization showing expression of MCK-10 in the tumor cells of an adenocarcinoma of the colon.

Figure 17. *In situ* hybridization showing expression of MCK-10 in the tumor cells of an adenocarcinoma of the colon.

Figure 18. *In situ* hybridization showing expression of MCK-10 in meningiothelial tumor cells.

Figure 19. *In situ* hybridization showing expression of MCK-10 in cells of a glioblastoma (glioma), a tumor of the neuroepithelial tissue.

Figure 20. *In situ* hybridization showing expression of MCK-10 in cells of a medulloblastoma with hyperchromatic atypical nuclei. Expression of MCK-10 is predominantly in cells with well developed cytoplasm.

Figure 21. *In situ* hybridization showing the expression of MCK-10 in cells of a medulloblastoma with hyperchromatic atypical nuclei. Expression of MCK-10 is predominantly in cells with well developed cytoplasm.

## 5. DETAILED DESCRIPTION

The present invention relates to a novel family of receptor tyrosine kinases referred to herein as MCK-10. The invention relates to differentially

spliced isoforms of MCK-10 and to additional members of the MCK-10 family of receptor tyrosine kinases such as the CCK-gene described herein. The invention is based, in part, on the isolation of a cDNA clone encoding the MCK-10 receptor tyrosine kinase and the discovery of differentially spliced isoforms of MCK-10. The invention also relates to the isolation of a cDNA encoding on additional member of MCK-10 receptor tyrosine kinase family, herein referred to as CCK-2.

Results from Northern Blot analysis and *in situ* hybridization indicates that MCK-10 is expressed in epithelial cells. In addition, MCK-10 expression can be detected in a wide variety of cancer cells lines and in all tested tumors. The invention relates to, expression and production of MCK-10 protein, as well as to inhibitors of MCK-10 receptor activity which may have therapeutic value in the treatment of diseases such as cancer.

For clarity of discussion, the invention is described in the subsections below by way of example for the MCK-10 gene depicted in Figures 1A, 1B and 1C and the CCK-2 gene depicted in Figures 3A, 3B, 3C and 3D. However, the principles may be analogously applied to differentially spliced isoforms of MCK-10 and to other members of the MCK-10 family of receptors.

#### 5.1. THE MCK-10 CODING SEQUENCE

The nucleotide coding sequence and deduced amino acid sequence of the human MCK-10 gene is depicted in Figures 1A, 1B and 1C (SEQ. ID NO. 1). In accordance with the invention, any nucleotide sequence which encodes the amino acid sequence of the MCK-10 gene product can be used to generate recombinant molecules which direct the expression of MCK-10. In additional embodiments of the invention, nucleotide sequences

which selectively hybridize to the MCK-10 nucleotide sequence shown in FIG. 1A, 1B and 1C (SEQ ID NO: 1) may also be used to express gene products with MCK-10 activity. Hereinafter all such variants of the MCK-10 nucleotide sequence will be referred to as the MCK-10 DNA sequence.

In a specific embodiment described herein, the human MCK-10 gene was isolated by performing a polymerase chain reaction (PCR) in combination with two degenerate oligonucleotide primer pools that were designed on the basis of highly conserved sequences within the kinase domain of receptor tyrosine kinases corresponding to the amino acid sequence HRDLAA (sense primer) and SDVWS/FY (antisense primer) (Hanks et al., 1988). As a template cDNA synthesized by reverse transcription of poly-A RNA from the human mammary carcinoma cell line MCF7, was used. A novel RTK, designated MCK-10 (mammary carcinoma kinase 10) was identified that within the tyrosine kinase domain exhibited extensive sequence similarity to the insulin receptor family. The PCR fragment was used to screen a lambda gt11 library of human fetal brain cDNA (Clontech). Several overlapping clones were identified. The composite of these cDNA clones is depicted in Figures 1A, 1B and 1C. Furthermore, screening of a human placental library yielded two cDNA clones, MCK-10-1 and MCK-10-2, which encoded the entire MCK-10 protein but contained a shorter 5' untranslated region starting at position 278 of the MCK-10 sequence (Figures 1A, 1B and 1C). Sequences analysis of the two clones revealed complete identity with the exception of 111 additional nucleotides within the juxtamembrane domain, between nucleotides 1832 and 1943. One of the clones isolated from the human fetal brain library contained an additional 18 nucleotides in the tyrosine kinase domain. These

sequences were in-frame with the MCK-10 open reading frame and did not contain any stop codons. The MCK-10 splice isoforms have been designated MCK-10-1 (with the additional 111 bp), MCK-10-2 (without any insertions), MCK-10-3 (with the additional 111 bp and 18 bp), and MCK-10-4 (with the additional 18 bp) (FIG. 2).

As shown in Figures 1A, 1B, and 1C and Figures 3A, 3B, 3C and 3D, MCK-10 have all of the characteristics of a receptor PTK: the initiation codon is followed by a stretch of essentially hydrophobic amino acids, which may serve as a signal peptide. Amino acids 417-439 are also hydrophobic in nature, with the characteristics of a transmembrane region. The extracellular domain encompasses 4 consensus N-glycosylation sites (AsnXSer/Thr) and 7 cysteine residues. The extracellular region is shorter than that of the insulin receptor family and shows no homology to other receptor tyrosine kinases, but contains near the N-terminus the consensus sequences for the discoidin I like family (Poole et al. 1981, J. Mol. Biol. 153: 273-289), which are located as tandem repeats in MGP and BA46, two milk fat globule membrane proteins (Stubbs et al. 1990, Proc. Natl. Acad. Sci. USA, 87, 8417-8421, Larocca et al. 1991, Cancer Res. 51: 4994-4998), in the light chains of factor V (Kane et al. 1986, Proc. Natl. Acad. Sci. USA, 83: 6800-6804) and VIII (Toole et al. 1984, Nature 312: 342-347), and in the A5 protein (Takagi et al. 1987, Dev. Biol., 122: 90-100).

The protein backbone of MCK-10-1 and MCK-10-2 proreceptors, with predicted molecular weights of 101.13 and 97.17 kD, respectively, can thus be subdivided into a 34.31 kD  $\alpha$  subunit and 66.84 or 62.88 kD  $\beta$ -subunits that contain the tyrosine kinase homology and alternative splice sites.

The consensus sequence for the ATP-binding motif is located at positions 617-627. When compared with other kinases, the ATP binding domain is with 176 amino acids (including the additional 37 amino acids) further from the transmembrane domain than any other tyrosine kinase. The additional 37 amino acids are located in the long and proline/glycine-rich juxtamembrane region and contain an NPAY sequence (where A can be exchanged for any amino acid), which is found in cytoplasmic domains of several cell surface proteins, including RTKs of the EGF and insulin receptor families (Chen et al. 1990, J. Biol. Chem., 265: 3116-3123). This consensus motif is followed by the sequence TYAXPXXXPG, which is repeated downstream in MCK-10 in the juxtamembrane domain at positions 585-595. Recently it has been shown that this motif is deleted in the cytoplasmic juxtamembrane region of the activin receptor, serine/threonine kinase, resulting in reduced ligand binding affinity (Attisano et al. 1992, Cell, 68: 97-108).

In comparison with other RTKs, the catalytic domain shows the highest homology to the TrkA receptor. The YY- motifs (position 802/803) and the tyrosine at position 798, representing putative autophosphorylation sites, characterize MCK-10 as a member of the insulin receptor family. Finally, MCK-10 shares homology with the Trk kinases with their characteristic short carboxyl-terminal tail of 9 amino acids.

To determine whether the additional 111 nucleotides present in MCK-10-1 and -3 were ubiquitously expressed or expressed only in specific human tissues, a PCR analysis on different human cDNAs using oligonucleotide primers corresponding to sequences flanking the insertion site was carried out. Parallel PCR amplifications were performed on plasmid

DNAs of MCK-10-1/MCK-10-2 as controls. Expression of both isoforms were identified in brain, pancreas, placenta, colon, and kidney, and in the cell lines Caki 2 (kidney ca), SW 48 (colon ca), and HBL100 and T47D (breast ca). The PCR products were subcloned into the Bluescript vector to confirm the nucleotide sequence.

Using a hybridization probe comprising the 5' 1694 bp cDNA fragment of MCK-10 (excluding the 111 bp insert), which encompasses the extracellular, transmembrane, and juxtamembrane domains, the MCK-10 gene revealed the existence of multiple transcript sizes with a major form of 4.2 kb. The highest expression of MCK-10 mRNA was detected in lung, intermediate levels were found in kidney, colon, stomach, placenta and brain, low levels in pancreas, and no MCK-10 mRNA was detected in liver (FIG. 5A). Figures 5B illustrates the levels of expression of MCK-10 in a variety of breast cancer cell lines and Figures 5C presents the levels of MCK-10 expression in different tumor cell lines. A summary of the expression patterns of MCK-10 in different cell lines is presented in TABLE 1.

TABLE 1

MCK-10 EXPRESSION IN DIFFERENT CELL LINES	
BREAST CANCER CELL LINES	
BT-474	+
T-47D	++++
BT-20	+++
MDA-MB-453	++
MDA-MB-468	++
MDA-MB-435	++
MDA-MB-175	++++

	MDA-MB-231	++
	HBL 100	+
	SK-BR-3	+
	MCF-7	++
5		
	<u>LUNG CANCER CELL LINES</u>	
	WI-38	+
	WI-26	+
10		
	<u>MELANOMA CELL LINES</u>	
	SK-Mel-3	+
	Wm 266-4	+
	HS 294T	++
15		
	<u>COLON CANCER CELL LINES</u>	
	Caco-2	+++
	-SNU-C2B	+++
	SW48	++
20		
	<u>KIDNEY CANCER CELL LINE</u>	
	CAKI-2	+++
	<u>EPIDERMOID CANCER CELL LINE</u>	
25	A431	++
	<u>OTHER CANCERS</u>	
	rhabdomyosarcoma	++
	Ewing sarcoma	++
	glioblastoma	++
30	neuroblastoma	-
	hepatoblastoma	+
	<u>HEMAPOIETIC CELL LINES</u>	
35	EB3	-
	CEM	-

5	MOLT4	-
	DAUDI	-
	RAJI	-
	MEG01	-
	KG1	-
	K562	-

10        *In situ* hybridization analysis with the 5' 1865 bp of MCK-10-2 indicated that MCK-10 was expressed specifically in epithelial cells of various tissues including:

- cuboidal epithelial cells lining the distal kidney tubulus (FIG. 7)
- 15 • columnar epithelial cells lining the large bowel tract
- deep layer of epithelial cells lining the stomach
- epithelial cells lining the mammary ducts
- islet cells of the pancreas (FIG. 13 and FIG. 14)
- 20 • epithelial cells of the thyroid gland, which produces thyroid hormones

No detectable MCK-10 expression was observed in connective tissues, endothelial cells, adipocytes, muscle cells, or hemopoietic cells.

25        MCK-10 expression was also detected in all tumors investigated which included:

- adenocarcinoma of the colon (FIG. 16 and FIG. 17)
- adenocarcinoma of the stomach
- 30 • adenocarcinoma of the lung
- infiltrating ductal carcinoma of the breast
- cystadenoma of the ovary
- multi endocrine tumor of the pancreas
- carcinoid tumor of the pancreas
- 35 • tubular cells of renal cell carcinoma

- transitional cell carcinoma (a malignant epithelial tumor of the bladder)
- meningiothelial tumor (FIG. 18)
- medulloblastoma with hyperchromatic atypical nuclei and spare cytoplasm (MCK-10 expression is only seen in cells with well developed cytoplasm) (FIG. 20 and FIG. 20)
- glioblastoma (a tumor of the neuroepithelial tissue) (FIG. 19)

10 The *in situ* hybridization experiments revealed the highest expression of MCK-10 in malignant cells of the ductal breast carcinoma, in the tumor cells of a multi-endocrine tumor, and in the tumor cells of a transitional cell carcinoma of the bladder.

#### 15 5.2 THE CCK-2 CODING SEQUENCE

The present invention also relates to other members of the MCK-10 family of receptor kinases. Members of the MCK-10 family are defined herein as those DNA sequences capable of hybridizing to MCK-10 DNA sequences as presented in Figures 1A, 1B and 1C. Such receptors may demonstrate 80% homology at the amino acid level in substantial stretches of DNA sequences. In addition, such receptors can be defined as those receptors containing an intracellular tyrosine kinase domain and a discoidin I sequence located near the amino-terminal end of the protein. The discoidin I domain is defined as that region of MCK-10 located between amino acid 31-185 as presented in Figure 1.

In a specific embodiment of the invention described herein, an additional member of the MCK-10 family of receptor tyrosine kinases was cloned and characterized. The nucleotide coding sequence and deduced amino acid sequence of the novel receptor

tyrosine kinase, herein referred to as CCK-2, is presented in Figures 3A, 3B, 3C and 3D. In accordance with the invention, any nucleotide sequence which encodes the amino acid sequence of the CCK-2 gene product can be used to generate recombinant molecules which direct the expression of CCK-2. In additional, embodiments of the invention, nucleotide sequences which selectively hybridize to the CCK-2 nucleotide sequence as shown in Figures 3A, 3B, 3C and 3D (SEQ. ID NO: 2) may also be used to express gene products with CCK-2 activity.

Analysis of the CCK-2 sequence revealed significant homology to the extracellular, transmembrane and intracellular region of the MCK-10 receptor indicating that it was a member of the MCK-10 family of receptors. The shared homology between CCK-2 and MCK-10 is depicted in Figure 4A and 4B.

### 5.3. EXPRESSION OF MCK-10 RECEPTOR AND GENERATION OF CELL LINES THAT EXPRESS MCK-10

For clarity of discussion the expression of receptors and generation of cell lines expressing receptors are described by way of example for the MCK-10 gene. However, the principles may be analogously applied to expression and generation of cell lines expressing spliced isoforms of MCK-10 or to other members of the MCK-10 family of receptors, such as CCK-2.

In accordance with the invention, MCK-10 nucleotide sequences which encode MCK-10, peptide fragments of MCK-10, MCK-10 fusion proteins or functional equivalents thereof may be used to generate recombinant DNA molecules that direct the expression of MCK-10 protein or a functionally equivalent thereof, in appropriate host cells. Alternatively,

nucleotide sequences which hybridize to portions of the MCK-10 sequence may also be used in nucleic acid hybridization assays, Southern and Northern blot analyses, etc.

5 Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used in the practice of the invention for the cloning and expression of the MCK-10 protein. Such DNA sequences include those which are capable of  
10 hybridizing to the human MCK-10 sequence under stringent conditions.

Altered DNA sequences which may be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues  
15 resulting in a sequence that encodes the same or a functionally equivalent gene product. These alterations would in all likelihood be in regions of MCK-10 that do not constitute functionally conserved regions such as the discordin I domain or the tyrosine  
20 kinase domain. In contrast, alterations, such as deletions, additions or substitutions of nucleotide residues in functionally conserved MCK-10 regions would possibly result in a nonfunctional MCK-10 receptor. The gene product itself may contain  
25 deletions, additions or substitutions of amino acid residues within the MCK-10 sequence, which result in a silent change thus producing a functionally equivalent MCK-10. Such amino acid substitutions may be made on the basis of similarity in polarity, charge,  
30 solubility, hydrophobicity, hydrophilicity, and/or the amphipatic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids  
35 with uncharged polar head groups having similar

hydrophilicity values include the following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine.

5 The DNA sequences of the invention may be engineered in order to alter the MCK-10 coding sequence for a variety of ends including but not limited to alterations which modify processing and expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, e.g. site-directed mutagenesis, to insert new  
10 restriction sites, to alter glycosylation patterns, phosphorylation, etc. For example, in certain expression systems such as yeast, host cells may over glycosylate the gene product. When using such expression systems it may be preferable to alter the MCK-10  
15 coding sequence to eliminate any N-linked glycosylation site.

In another embodiment of the invention, the MCK-10 or a modified MCK-10 sequence may be ligated to a heterologous sequence to encode a fusion protein. For  
20 example, for screening of peptide libraries it may be useful to encode a chimeric MCK-10 protein expressing a heterologous epitope that is recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located  
25 between the MCK-10 sequence and the heterologous protein sequence, so that the MCK-10 may be cleaved away from the heterologous moiety.

In an alternate embodiment of the invention, the coding sequence of MCK-10 could be synthesized in  
30 whole or in part, using chemical methods well known in the art. See, for example, Caruthers, et al., 1980, Nuc. Acids Res. Symp. Ser. 7:215-233; Crea and Horn, 180, Nuc. Acids Res. 9(10):2331; Matteucci and Caruthers, 1980, Tetrahedron Letters 21:719; and Chow  
35 and Kempe, 1981, Nuc. Acids R s. 9(12):2807-2817.

Alternatively, the protein in its full length could be produced using chemical methods to synthesize the MCK-10 amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography. (E.g., see Creighton, 1983, Proteins Structures And Molecular Principles, W.H. Freeman and Co., N.Y. pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, 1983, Proteins, Structures and Molecular Principles, W.H. Freeman and Co., N.Y., pp. 34-49).

In order to express a biologically active MCK-10, the nucleotide sequence coding for MCK-10, or a functional equivalent, is inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. The MCK-10 gene products as well as host cells or cell lines transfected or transformed with recombinant MCK-10 expression vectors can be used for a variety of purposes. These include but are not limited to generating antibodies (i.e., monoclonal or polyclonal) that bind to the receptor, including those that competitively inhibit binding of MCK-10 ligand and "neutralize" activity of MCK-10 and the screening and selection of drugs that act via the MCK-10 receptor; etc.

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#### 5.3.1. EXPRESSION SYSTEMS

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the MCK-10 coding sequence and appropriate transcriptional/translational control signals. These methods include in vitro recombinant DNA techniques,

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synthetic techniques and in vivo recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.

A variety of host-expression vector systems may be utilized to express the MCK-10 coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the MCK-10 coding sequence; yeast transformed with recombinant yeast expression vectors containing the MCK-10 coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the MCK-10 coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the MCK-10 coding sequence; or animal cell systems. The expression elements of these systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage  $\lambda$ , plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters;

the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters  
5 derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the MCK-10 DNA,  
10 SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the MCK-10 expressed. For  
15 example, when large quantities of MCK-10 are to be produced for the generation of antibodies or to screen peptide libraries, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors  
20 include but are not limited to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the MCK-10 coding sequence may be ligated into the vector in frame with the lac Z coding region so that a hybrid AS-lac Z protein is produced; pIN  
25 vectors (Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In  
30 general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease  
35

cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety.

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II.

In cases where plant expression vectors are used, the expression of the MCK-10 coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J. 3:1671-1680; Broglie et al., 1984, Science 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-

463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9.

An alternative expression system which could be used to express MCK-10 is an insect system. In one such system, Autographa californica nuclear polyhydrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The MCK-10 coding sequence may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the MCK-10 coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed. (E.g., see Smith et al., 1983, J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the MCK-10 coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing MCK-10 in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659). Alternatively, the vaccinia 7.5K promoter may be used. (See, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett et al., 1984, J. Virol.

49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. 79:4927-4931).

Specific initiation signals may also be required for efficient translation of inserted MCK-10 coding sequences. These signals include the ATG initiation  
5 codon and adjacent sequences. In cases where the entire MCK-10 gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases  
10 where only a portion of the MCK-10 coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the MCK-10 coding sequence  
15 to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription  
20 enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted  
25 sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. The presence of four consensus N-  
30 glycosylation sites in the MCK-10 extracellular domain support that proper modification may be important for MCK-10 function. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins.  
35 Appropriate cells lines or host systems can be chosen

to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the MCK-10 may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the MCK-10 DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the MCK-10 on the cell surface. Such engineered cell lines are particularly useful in screening for drugs that affect MCK-10.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase

(Lowy, et al., 1980, Cell 22:817) genes can be employed in tk, hgp<sup>r</sup>t or ap<sup>r</sup>t cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hyg<sup>r</sup>, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

5.3.2. IDENTIFICATION OF TRANSFECTANTS  
OR TRANSFORMANTS THAT EXPRESS THE  
MCK-10

The host cells which contain the coding sequence and which express the biologically active gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of MCK-10 mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

In the first approach, the presence of the MCK-10 coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the MCK-10 coding sequence,  
5 respectively, or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity,  
10 resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the MCK-10 coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the MCK-10 coding  
15 sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the MCK-10 sequence under the control of the same or different promoter used to control the expression of the MCK-10 coding sequence.  
20 Expression of the marker in response to induction or selection indicates expression of the MCK-10 coding sequence.

In the third approach, transcriptional activity for the MCK-10 coding region can be assessed by  
25 hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the MCK-10 coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for  
30 hybridization to such probes.

In the fourth approach, the expression of the MCK-10 protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation,  
35 enzyme-link d immunoassays and the like.

5.4. USES OF THE MCK-10 RECEPTOR  
AND ENGINEERED CELL LINES

For clarity of discussion the uses of the expressed receptors and engineered cell lines expressing the receptors is described by way of example for MCK-10. The described uses may be equally applied to expression of MCK-10 spliced isoforms or additional members of the MCK-10 gene family such as CCK-2.

In an embodiment of the invention the MCK-10 receptor and/or cell lines that express the MCK-10 receptor may be used to screen for antibodies, peptides, or other ligands that act as agonists or antagonists of the MCK-10 receptor. For example, anti-MCK-10 antibodies may be used to inhibit MCK-10 function. Alternatively, screening of peptide libraries with recombinantly expressed soluble MCK-10 protein or cell lines expressing MCK-10 protein may be useful for identification of therapeutic molecules that function by inhibiting the biological activity of MCK-10. The uses of the MCK-10 receptor and engineered cell lines, described in the subsections below, may be employed equally well for MCK-10 family of receptor tyrosine kinases.

In an embodiment of the invention, engineered cell lines which express the entire MCK-10 coding region or its ligand binding domain may be utilized to screen and identify ligand antagonists as well as agonists. Synthetic compounds, natural products, and other sources of potentially biologically active materials can be screened in a number of ways.

#### 5.4.1. SCREENING OF PEPTIDE LIBRARY WITH MCK-10 PROTEIN OR ENGINEERED CELL LINES

Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to bind to the ligand binding site of a given receptor or other functional domains of a receptor such as kinase domains (Lam, K.S. et al., 1991, Nature 354: 82-84). The screening of peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that act to inhibit the biological activity of receptors through their interactions with the given receptor.

Identification of molecules that are able to bind to the MCK-10 may be accomplished by screening a peptide library with recombinant soluble MCK-10 protein. Methods for expression and purification of MCK-10 are described in Section 5.2.1 and may be used to express recombinant full length MCK-10 or fragments of MCK-10 depending on the functional domains of interest. For example, the kinase and extracellular ligand binding domains of MCK-10 may be separately expressed and used to screen peptide libraries.

To identify and isolate the peptide/solid phase support that interacts and forms a complex with MCK-10, it is necessary to label or "tag" the MCK-10 molecule. The MCK-10 protein may be conjugated to enzymes such as alkaline phosphatase or horseradish peroxidase or to other reagents such as fluorescent labels which may include fluorescein isothiocyanate (FITC), phycoerythrin (PE) or rhodamine. Conjugation of any given label, to MCK-10, may be performed using techniques that are routine in the art.

Alternatively, MCK-10 expression vectors may be engineered to express a chimeric MCK-10 protein

containing an epitope for which a commercially available antibody exist. The epitope specific antibody may be tagged using methods well known in the art including labeling with enzymes, fluorescent dyes or colored or magnetic beads.

- 5       The "tagged" MCK-10 conjugate is incubated with the random peptide library for 30 minutes to one hour at 22°C to allow complex formation between MCK-10 and peptide species within the library. The library is then washed to remove any unbound MCK-10 protein. If
- 10   MCK-10 has been conjugated to alkaline phosphatase or horseradish peroxidase the whole library is poured into a petri dish containing substrates for either alkaline phosphatase or peroxidase, for example, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) or
- 15   3,3',4,4"-diamnobenzidine (DAB), respectively. After incubating for several minutes, the peptide/solid phase-MCK-10 complex changes color, and can be easily identified and isolated physically under a dissecting microscope with a micromanipulator. If a fluorescent
- 20   tagged MCK-10 molecule has been used, complexes may be isolated by fluorescent activated sorting. If a chimeric MCK-10 protein expressing a heterologous epitope has been used, detection of the peptide/MCK-10 complex may be accomplished by using a labeled
- 25   epitope specific antibody. Once isolated, the identity of the peptide attached to the solid phase support may be determined by peptide sequencing.

- In addition to using soluble MCK-10 molecules, in another embodiment, it is possible to detect peptides
- 30   that bind to cell surface receptors using intact cells. The use of intact cells is preferred for use with receptors that are multi-subunits or labile or with receptors that require the lipid domain of the cell membrane to be functional. Methods for
- 35   generating cell lines expressing MCK-10 are described

in Sections 5.2.1. and 5.2.2. The cells used in this technique may be either liv or fixed cells. The cells will be incubated with the random peptide library and will bind to certain peptides in the library to form a "rosette" between the target cells and the relevant solid phase support/peptide. The rosette can thereafter be isolated by differential centrifugation or removed physically under a dissecting microscope.

As an alternative to whole cell assays for membrane bound receptors or receptors that require the lipid domain of the cell membrane to be functional, the receptor molecules can be reconstituted into liposomes where label or "tag" can be attached.

#### 5.4.2. ANTIBODY PRODUCTION AND SCREENING

Various procedures known in the art may be used for the production of antibodies to epitopes of the recombinantly produced MCK-10 receptor. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library. Neutralizing antibodies i.e., those which compete for the ligand binding site of the receptor are especially preferred for diagnostics and therapeutics.

Monoclonal antibodies that bind MCK-10 may be radioactively labeled allowing one to follow their location and distribution in the body after injection. Radioactivity tagged antibodies may be used as a non-invasive diagnostic tool for imaging *de novo* cells of tumors and metastases.

Immunotoxins may also be designed which target cytotoxic agents to specific sites in the body. For example, high affinity MCK-10 specific monoclonal antibodies may be covalently complexed to bacterial or plant toxins, such as diphtheria toxin, abrin or

ricin. A general method of preparation of antibody/hybrid molecules may involve use of thiol-crosslinking reagents such as SPDP, which attack the primary amino groups on the antibody and by disulfide exchange, attach the toxin to the antibody. The  
5 hybrid antibodies may be used to specifically eliminate MCK-10 expressing tumor cells.

For the production of antibodies, various host animals may be immunized by injection with the MCK-10 protein including but not limited to rabbits, mice,  
10 rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as  
15 lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacilli Calmette-Guerin) and Corynebacterium parvum.

20 Monoclonal antibodies to MCK-10 may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by  
25 Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and  
30 Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985,  
35 Nature, 314:452-454) by splicing the genes from a

mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce MCK-10-specific single chain antibodies.

Antibody fragments which contain specific binding sites of MCK-10 may be generated by known techniques. For example, such fragments include but are not limited to: the  $F(ab')_2$  fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the  $F(ab')_2$  fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to MCK-10.

#### 5.5. USES OF MCK-10 CODING SEQUENCE

The MCK-10 coding sequence may be used for diagnostic purposes for detection of MCK-10 expression. Included in the scope of the invention are oligoribonucleotide sequences, that include antisense RNA and DNA molecules and ribozymes that function to inhibit translation of MCK-10. In addition, mutated forms of MCK-10, having a dominant negative effect, may be expressed in targeted cell populations to inhibit the activity of endogenously expressed MCK-10. The uses described below may be equally well adapted for MCK-10 spliced isoform coding sequences and sequences encoding additional members of the MCK-10 family of receptors, such as CCK-2.

5.5.1. USE OF MCK-10 CODING SEQUENCE  
IN DIAGNOSTICS AND THERAPEUTICS

The MCK-10 DNA may have a number of uses for the diagnosis of diseases resulting from aberrant expression of MCK-10. For example, the MCK-10 DNA  
5 sequence may be used in hybridization assays of biopsies or autopsies to diagnose abnormalities of MCK-10 expression; e.g., Southern or Northern analysis, including *in situ* hybridization assays.

Also within the scope of the invention are oligo-  
10 ribonucleotide sequences, that include anti-sense RNA and DNA molecules and ribozymes that function to inhibit the translation of MCK-10 mRNA. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and  
15 preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the MCK-10 nucleotide sequence, are preferred.

20 Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage.  
25 age. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of MCK-10 RNA sequences.

Specific ribozyme cleavage sites within any  
30 potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of  
35 between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site

may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

5.5.2. USE OF DOMINANT NEGATIVE  
MCK-10 MUTANTS IN GENE THERAPY

Receptor dimerization induced by ligands, is thought to provide an allosteric regulatory signal that functions to couple ligand binding to stimulation of kinase activity. Defective receptors can function as dominant negative mutations by suppressing the activation and response of normal receptors by formation of unproductive heterodimers. Therefore, defective receptors can be engineered into recombinant viral vectors and used in gene therapy in individuals that inappropriately express MCK-10.

In an embodiment of the invention, mutant forms of the MCK-10 molecule having a dominant negative effect may be identified by expression in selected cells. Deletion or missense mutants of MCK-10 that retain the ability to form dimers with wild type MCK-10 protein but cannot function in signal transduction may be used to inhibit the biological activity of the endogenous wild type MCK-10. For example, the cytoplasmic kinase domain of MCK-10 may be deleted resulting in a truncated MCK-10 molecule that is still able to undergo dimerization with endogenous wild type receptors but unable to transduce a signal.

Recombinant viruses may be engineered to express dominant negative forms of MCK-10 which may be used to inhibit the activity of the wild type endogenous MCK-10. These viruses may be used therapeutically for treatment of diseases resulting from aberrant expression or activity of MCK-10, such as cancers.

Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of recombinant MCK-10 into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct

those recombinant viral vectors containing MCK-10 coding sequence. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. Alternatively, recombinant MCK-10 molecules can be reconstituted into liposomes for delivery to target cells.

10                   6.   EXAMPLES: CLONING AND CHARACTERIZATION OF MCK-10

                  The subsection below describes the isolation and characterization of a cDNA clones encoding the novel receptor tyrosine kinase designated MCK-10 and differentially spliced isoforms of MCK-10.

                  6.1.   MATERIALS AND METHODS

20                   6.1.1.   cDNA CLONING AND CHARACTERIZATION OF MCK-10

                  Confluent plates of the human breast cancer cell line MCF7 (American Type Culture Collection HTB22) were lysed by treatment with guanidinium-thiocyanate according to Chirgwin et al. (1979, Biochemistry 18:5294-5299). Total RNA was isolated by CsCl-gradient centrifugation. First-strand cDNA was synthesized from 20 µg total RNA with avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer Mannheim).

30                   cDNA was used in a polymerase chain reaction under standard conditions (*PCR Technology-Principles and Applications for DNA Amplifications*, H.E. Erlich, ed., Stockton Press, New York 1989). The following pool of primers were used for the amplification:

35

Sense Primer

corresponding to the amino acid sequence HRDLAA

EcoRI

5' GGAATTCC CAC AGN GAC TTN GCN GCN AG 3'  
T C A T C A A C

5

Antisense Primer

corresponding to the amino acid sequence SDVWS  
F/Y

EcoRI

10 3' TCN GAC GTN TGG ACN TTC CCTTAAGG 5'  
G G TG CAT

Thirty-five PCR cycles were carried out using  
8 µg (0.8 µg) of the pooled primers. (Annealing 55°C,  
15 1 min; Extension 72°C, 2 min; Denaturation 94°C, 1  
min). The reaction product was subjected to  
polyacrylamide gel electrophoresis. Fragments of the  
expected size (~210 bp) were isolated, digested with  
the restriction enzyme EcoRI, and subcloned into the  
20 pBluescript vector (Stratagene) using standard  
techniques (*Current Protocols in Molecular Biology*,  
eds. F.M. Ausubel et al., John Wiley & Sons, New York,  
1988).

25 The recombinant plasmids were transformed into  
the competent E. coli strain designated 298.

The subcloned PCR products were sequenced by the  
method of Sanger et al. (Proc. Natl. Acad. Sci. USA  
74, 5463-5467) using Sequenase (United States  
Biochemical, Cleveland, Ohio 44111 USA). One clone,  
30 designated MCK-10 was identified as novel RTK.

6.1.2. FULL-LENGTH CDNA CLONING

The partial cDNA sequence of the new MCK-10 RTK,  
which was identified by PCR, was used to screen a  
35 λgt11 library from human fetal brain cDNA (Clontech)

(complexity of  $1 \times 10^{10}$  recombinant phages). One million independent phage clones were plated and transferred to nitrocellulose filters following standard procedures (Sambrook, H.J., Molecular Cloning, Cold Spring Harbor Laboratory Press, USA, 1989). The filters were hybridized to the EcoRI/EcoRI fragment of clone MCK-10, which had been radioactively labeled using  $50 \mu\text{Ci}$  [ $\alpha^{32}\text{P}$ ]ATP and the random-primed DNA labeling kit (Boehringer Mannheim). The longest cDNA insert (8) of ~3500 bp was digested with the restriction enzymes EcoRI/SacI to obtain a 5' end probe of 250 bp. This probe was used to rescreen the human fetal brain library and several overlapping clones were isolated. The composite of the cDNA clones are shown in Figures 1A, 1B and 1C. Some of the clones had a deletion of 6 amino acids at position 2315 in the MCK-10 sequence.

The 1.75 million independent phage clones of a human placenta library,  $\lambda$ ZAP were plated and screened with the 5' end probe (EcoRI/SacI) of clone 8. Two clones were full-length with a shorter 5' end starting at position 278 of the nucleotide sequence shown in Figures 1A, 1B and 1C. Subcloning of positive bacteriophages clones into pBluescript vector was done by the *in vivo* excision protocol (Stratagene).

The composite cDNA sequence and the predicted amino acid sequence of MCK-10 are shown in Figures 1A, 1B, and 1C. Different cDNA sequence variations of MCK-10 is presented in Figure 2.

#### 6.1.3. NORTHERN BLOT ANALYSIS OF MCK-10

Total RNA was isolated from the following human tissues: lung, pancreas, stomach, kidney, spleen, liver, colon and placenta. RNA was also isolated from various breast cancer cell lines and cell lines of tumor origin.

PolyA<sup>+</sup> RNA was isolated on an oligo (dT) column (Aviv and Leder, 1972, Proc. Natl. Acad. Sci. USA 69, 1408-1412). The RNA was separated on an agarose gel containing 2.2M formaldehyde and blotted on a nitrocellulose filter (Schleicher and Schuell). 3μg of poly A<sup>+</sup> RNA was loaded per lane. The filter was hybridized with a <sup>32</sup>P-labeled EcoRI/EcoRI DNA fragment obtained by PCR. Subsequently, the filter was exposed to x-ray film at -70°C with an intensifying screen. The results are depicted in Figures 5A, 5B and 5C.

#### 6.1.4. GENERATION OF MCK-10 SPECIFIC ANTIBODIES

Antisera was generated against synthetic peptides corresponding to the amino acid sequence of MCK-10.

αMCK-10-N antisera was generated against the following N-terminal peptide located between amino acids 26-42:

H-F-D-P-A-K-D-C-R-Y-A-L-G-M-Q-D-R-T-I.

αMCK-10-c antisera was generated against the following C-terminal peptide located between amino acids 902-919

R-P-P-F-S-Q-L-H-R-F-L-A-E-D-A-L-N-T-V.

αMCK-10-β antisera was generated against the following peptide near the processing site of β-subunit of MCK-10 located between amino acids 309-322:

P-A-M-A-W-E-G-E-P-M-R-H-N-L.

αMCK-10-C2 antisera was generated against the C-terminal peptide located between amino acids 893-909:

C-W-S-R-E-S-E-Q-R-P-P-F-S-Q-L-H-R.

Peptides were coupled to keyhole limpet hemocyanin and injected with Freund's adjuvant into Chinchilla rabbits. After the second boost, the rabbits were bled and the antisera were tested in immunoprecipitations using lysates of 293 cells transiently overexpressing MCK-10-1 and MCK-10-2.

The samples were loaded on a 7.5% polyacrylamide gel and after electrophoresis transferred onto a nitrocellulose filter (Schleicher and Schuell). The blot was probed with the different antibodies as above and developed using the ECL Western blotting detection system according to the manufacturer's instructions (Cat no. RPN 2108 Amersham International, UK).

#### 6.1.5. IN SITU HYBRIDIZATION

The 5' located cDNA fragment corresponding to nucleotides 278-1983 of clone MCK-10, excluding the 111 base pair insert, were subcloned in the bluescript SK+ (Stratagene). For *in situ* hybridization, a single-strand antisense DNA probe was prepared as described by Schnürch and Risau (Development 1991, 111, 1143-1154). The plasmid was linearized at the 3' end of the cDNA and a sense transcript was synthesized using SP6 RNA polymerase (Boehringer). The DNA was degraded using DNase (RNase-free preparation, Boehringer Mannheim). With the transcript, a random-primed cDNA synthesis with  $\alpha$ -<sup>35</sup>S ATP (Amersham) was performed by reverse transcription with MMLV reverse transcriptase (BRL). To obtain small cDNA fragments of about 100 bp in average, suitable for *in situ* hybridization, a high excess of primer was used. Subsequently, the RNA transcript was partially hydrolyzed in 100 nM NaOH for 20 min at 70°C, and the probe was neutralized with the same amount of HCL and purified with a Sephadex-G50 column. After ethanol precipitation the probe was dissolved at a final specific activity of  $5 \times 10^5$  cpm. For control hybridization, a sense probe was prepared using the same method.

Sectioning, postfixation was essentially performed according to Hogan et al. (1986, Manipulating the Mouse Embryo: A Laboratory Manual,

New York: Cold Spring Harbor Laboratory Press). 10  $\mu$ m thick sections were cut at  $-18^{\circ}\text{C}$  on a Leitz cryostat. For hybridization treatment, no incubation with 0.2M HCL for removing the basic proteins was performed. Sections were incubated with the  $^{35}\text{S}$ -cDNA probe ( $5 \times 10^4 \text{cpm}/\mu\text{l}$ ) at  $52^{\circ}\text{C}$  in a buffer containing 50% formamide, 300mM NaCl, 10 mM Tris-HCL, 10mM  $\text{NaPO}_4$  (pH 6.8), 5mM EDTA, 2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.02% BSA, 10 mg/ml yeast RNA, 10% dextran sulfate, and 10mM DTT. Posthybridization washing was performed at high stringency (50% formamide, 300mM NaCl, 10mM Tris-HCL, 10 mM  $\text{NaPO}_4$  (pH6.8), 5mM EDTA, 10 mM DTT at  $52^{\circ}\text{C}$ ). For autoradiography, slides were created with Kodak NTB2 film emulsion and exposed for eight days. After developing, the sections were counterstained with toluidine blue.

## 6.2. RESULTS

### 6.2.1. CHARACTERIZATION OF MCK-10 CLONE

To identify novel receptor tyrosine kinases (RTKs) that are expressed in mammary carcinoma cell lines, we used the polymerase chain reaction in combination with two degenerate oligonucleotide primer pools based on highly conserved sequences within the kinase domain of RTKs, corresponding to the amino acid sequence HRDLAA (sense primer) and SDVWS/FY (antisense primer) (Hanks et al. 1988, Science 241, 42-52), in conjunction with cDNA synthesized by reverse transcription of poly A RNA from the human mammary carcinoma cell line MCF7. We identified a novel RTK, designated MCK-10 (mammary carcinoma kinase 10), that within the tyrosine kinase domain exhibited extensive sequence similarity to the insulin receptor family. The PCR fragment was used to screen a lambda gt11 library of human fetal brain cDNA (Clontech). Several

overlapping clones were identified and their composite sequence is shown in Figures 1A, 1B and 1C.

Furthermore, screening of a human placenta library yielded two cDNA clones which encoded the entire MCK-10 protein but whose 5' nucleotide sequence began at  
5 nucleotide 278 in the sequence shown in Figure 1.

Sequence analysis of the two clones revealed complete identity with the exception of 111 additional nucleotides within the juxtamembrane domain, between nucleotides 1832 and 1943. One of the clones isolated  
10 from the human fetal brain library contained an additional 18 nucleotides in the tyrosine kinase domain. These sequences were in-frame with the MCK-10 open reading frame and did not contain any stop codons. We designated these MCK-10 splice isoforms  
15 MCK-10-1 (with the additional 111 bp, MCK-10-2 (without any insertions), MCK-10-3 (with the additional 111 bp and 18 bp), and MCK-10-4 (with the additional 18 bp). This new receptor tyrosine kinase was recently described by Johnson et al. (1993, Proc.  
20 Natl. Acad. Sci. USA, 90 5677-5681) as DDR.

As shown in Figure 1, MCK-10 has all of the characteristics of a receptor PTK: the initiation codon is followed by a stretch of essentially hydrophobic amino acids, which may serve as a signal  
25 peptide. Amino acids 417-439 are also hydrophobic in nature, with the characteristics of a transmembrane region. The extracellular domain encompasses 4 consensus N-glycosylation sites (AsnXSer/Thr) and 7 cysteine residues. The extracellular region is  
30 shorter than that of the insulin receptor family and shows no homology to other receptor tyrosine kinases, but contains near the N-terminus the consensus sequences for the discoidin 1 like family (Poole et al. 1981, J. Mol. Biol. 153, 273-289), which are  
35 located as tandem repeats in MGP and BA46, two milk

fat globule m mbrane proteins (Stubbs et al. 1990, proc. Natl. Acad. Sci. USA, 87, 8417-8421, Larocca et al. 1991, Cancer Res. 51, 4994-4998), in the light chains of factor V (Kane et al. 1986, Proc. Natl. Acad. Sci. USA, 83, 6800-6804) and VIII (Toole et al. 1984, Nature, 312, 342-347), and in the A5 protein (Takagi et al. 1987, Dev. Biol., 122, 90-100).

The protein backbone of MCK-10-1 and MCK-10-2 proreceptors, with predicted molecular weights of 101.13 and 97.17kD, respectively, can thus be subdivided into a 34.31 kD  $\alpha$  subunit and 66.84 kD  $\beta$ -subunits that contain the tyrosine kinase homology and alternative splice sites.

The consensus sequence for the ATP-binding motif is located at positions 617-627. When compared with other kinases, the ATP binding domain is 176 amino acids (including the additional 37 amino acids) further from the transmembrane domain than any other tyrosine kinase. The additional 37 amino acids are located in the long and proline/glycine-rich juxtamembrane region and contain an NPAY sequence (where A can be exchanged for any amino acid), which is found in cytoplasmic domains of several cell surface proteins, including RTKs of the EGF and insulin receptor families (Chen et al. 1990, J. Biol. Chem., 265, 3116-3123). This consensus motif is followed by the sequence TYAXPXXXPG, which is repeated downstream in MCK-10 in the juxtamembrane domain at positions 585-595. Recently it has been shown that this motif is deleted in the cytoplasmic juxtamembrane region of the activin receptor, a serine/threonine kinase, resulting in reduced ligand binding affinity (Attisano et al. 1992, Cell, 68, 97-108).

In comparison with other RTKs, the catalytic domain shows the highest homology to the TrkA receptor. The yy- motifs (position 802/803) and the

tyrosine at position 798, representing putative autophosphorylation sites, characterize MCK-10 as a member of the insulin receptor family. Finally, MCK-10 shares with the Trk kinases their characteristic short carboxy-terminal tail of 9 amino acids.

5 To determine whether the additional 111 nucleotides present in MCK-10-1 and -3 were ubiquitously expressed or expressed only in specific human tissues, we performed PCR on different human cDNAs using oligonucleotide primers corresponding to  
10 sequences flanking the insertion site. Parallel PCR amplifications were performed on plasmid DNAs of MCK-10-1/MCK-10-2 as controls. Expression of both isoforms was identified in brain, pancreas, placenta, colon, and kidney, and in the cell lines Caki 2  
15 (kidney ca), SW 48 (colon ca), and HBL100 and T47D (breast ca). The PCR products were subcloned into the Bluescript vector to confirm the nucleotide sequence.

20 6.2.2. NORTHERN BLOT ANALYSIS: EXPRESSION OF MCK-10 IN VARIOUS HUMAN TISSUES AND CELL LINES

Using as a hybridization probe a 5' 1694 bp cDNA fragment of MCK-10 (excluding the 111 base pair insert), which encompasses the extracellular,  
25 transmembrane, and juxtamembrane domains, the MCK-10 gene revealed the existence of multiple transcript sizes with a major form of 4.2 kb. The highest expression of MCK-10 mRNA was detected in lung, intermediate levels were found in kidney, colon,  
30 stomach, placenta, and brain, low levels in pancreas, and no MCK-10 mRNA was detected in liver (FIG. 5A). MCK-10 mRNA was also detected in a variety of different tumor cell lines as depicted in Figure 5B and Figure 5C. Northern blot analysis with the GAPDH  
35 gene was carried out as a control.

### 6.2.3. IN SITU HYBRIDIZATION

To determine which cells in the different human tissues contain MCK-10 transcripts, *in situ* hybridization of various human tissues and of tissues of different tumors were carried out. Hybridization analyses with the 5' 1694 bp of MCK-10 (excluding the 111 base pair insert) indicated that MCK-10 expression was specifically detected in epithelial cells of various tissues:

- cuboidal epithelial cells lining the distal kidney tubulus
- columnar epithelial cells lining the large bowel tract
- deep layer of epithelial cells lining the stomach
- epithelial cells lining the mammary ducts
- islet cells of the pancreas
- epithelial cells of the thyroid gland, which produces thyroid hormones

No detectable MCK-10 expression was observed in connective tissues, endothelial cells, adipocytes, muscle cells, or hemopoietic cells.

MCK-10 expression was detected in all tumors investigated:

- adenocarcinoma of the colon
- adenocarcinoma of the stomach
- adenocarcinoma of the lung
- infiltrating ductal carcinoma of the breast
- cystadenoma of the ovary
- multi endocrine tumor of the pancreas
- carcinoid tumor of the pancreas
- tubular cells of renal cell carcinoma
- transitional cell carcinoma (a malignant epithelial tumor of the bladder)
- meningiothelial tumor

medulloblastoma with hyperchromatic atypical nuclei and spare cytoplasm (MCK-10 expression is only seen in cells with well developed cytoplasm)

- glioblastoma (a tumor of the neuroepithelial tissue)

5        These *in situ* hybridization experiments revealed the highest expression of MCK-10 in malignant cells of the ductal breast carcinoma, in the tumor cells of a multi endocrine tumor, and in the tumor cells of a transitional cell carcinoma of the bladder. The *in*  
10 *situ* hybridization results are depicted in Figures 7-21.

#### 6.2.4. TRANSIENT OVEREXPRESSION OF MCK-10 IN 293 CELLS

15        To analyze the MCK-10 protein in detail, we used the 293 cell system for transient overexpression. The cDNAs of MCK-10-1 and MCK-10-2 were cloned into an expression vector. Cells were transfected in duplicate with the two splice variants or a control  
20 plasmid and starved overnight. One part was incubated prior to lysis with 1 mM sodium-orthovanadate for 90 min. This agent is known to be a potent inhibitor of phosphotyrosine phosphatases, thereby enhancing the tyrosine phosphorylation of cellular protein.

25        The precursor and the  $\beta$ -subunit of MCK-10 showed strong tyrosine phosphorylation after orthovanadate treatment, (FIG. 4A, left panel). Surprisingly, the MCK-10-1, containing the 37 amino acid insertion, exhibited lower kinase activity than MCK-10-2.  
30 Reprobing the same blot with a peptide antibody raised against the MCK-10 C-terminus revealed equal amounts of expressed receptor and a slight shift of MCK-10-1 precursor and  $\beta$ -subunit due to the additional 37 amino acids of the insertion (FIG. 4A, right panel).  
35

We further analyzed the N-linked glycosylation of the splice variants. Transfected cells were treated overnight with tunicamycin, which inhibits the maturation of proteins by glycosylation. Two affinity purified antibodies raised against peptide sequence of MCK-10 N- and C-terminus, respectively, were used for subsequent immunoprecipitations. Both antibodies precipitated the predicted 101 kD or 97 kD polypeptides from tunicamycin-treated cells (FIG. 4B). Interestingly, the size of the fully glycosylated forms of MCK-10-1 and MCK-10-2 suggested that the latter was more extensively glycosylated than the putative alternative splice form. This data indicates that the 37 amino acid insertion of MCK-10-1 influences its posttranslational modification which may influence ligand.

#### 7. EXAMPLES: CLONING AND CHARACTERIZATION OF CCK-2

The following subsection describes methods for isolation and characterization of the CCK-2 gene, an additional member of the MCK-10 receptor tyrosine kinase gene family.

##### 7.1. MATERIALS AND METHODS

##### 7.1.1. CDNA CLONING AND CHARACTERIZATION OF CCK-2

cDNA was synthesized using avian myeloblastosis virus reverse transcriptase and 5  $\mu$ g of poly A<sup>+</sup> RNA prepared from tissue of a primary colonic adenocarcinoma, sigmoid colon, moderately well differentiated grade II, staging pT3, pN1, removed from a 69 year old white female of blood type O, RH positive. The patient had not received therapy.

The tissue was minced and lysed by treatment with guanidinium-thiocyanate according to Chirgwin, J.M. et

al. (1979, Biochemistry 18:5294-5299). Total RNA was isolated by guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski et al. 1987, Anal. Biochem. 162:156-159). Poly A<sup>+</sup> RNA was isolated on an oligo-dT column (Aviv and Leder, 1972, Proc. Natl. Acad. Sci. USA 69:1408-1412).

One tenth of the cDNA was subjected to the polymerase chain reaction using standard conditions (PCR Technology- Principles and Applications for DNA Amplifications, H.E. Erlich, ed. Stockton Press, New York, 1989) and the same pool of primers used for amplification of MCK-10 (See, Section 6.1.1., lines 4-16). Thirty-five cycles were carried out (Annealing 55°C, 1 min; Extension 72°C, 2 min; Denaturation 94°C, 1 min.). The reaction products were subjected to polyacrylamide gel electrophoresis. Fragments of the expected size were isolated, digested with the restriction enzyme EcoRI, and subcloned into pBluescript vector (Stratagene) using standard techniques (Current Protocols in Molecules Biology, eds. M. Ausubel et al., John Wiley & Sons, New York, 1988). The subcloned PCR products were sequenced by the method of Sanger et al. (Proc. Natl. Acad. Sci. USA 74, 5463-5467) using T7-Polymerase (Boehringer Mannheim).

The CCK-2 PCR fragment was used to screen a human placenta library in lambda ZAP. The longest cDNA insert ~1300 bp was digested with the restriction enzymes EcoRI/NcoI to obtain a 5' end probe of 200 bp. Rescreening of the human placenta library yielded in a cDNA clone which encoded the entire CCK-2 protein (subcloning of positive bacteriophages clones into pBluescript vector was done by the *in vivo* excision protocol (Stratagene)). The DNA sequence and the deduced amino acid sequence of CCK-2 is shown in Figur 3.

## 7.2. RESULTS

### 7.2.1. CLONING AND CHARACTERIZATION OF CCK-2

An additional member of the MCK-10 receptor tyrosine kinase family was identified using a  
5 polymerase chain reaction and cDNA prepared from  
colonic adenocarcinoma RNA. The nucleotide sequence  
of the novel receptor, designated CCK-2, is presented  
in Figures 3A and 3B. Analysis of the CCK-2,  
10 nucleotide sequence and encoded amino acid sequence  
indicated significant homology with MCK-10 throughout  
the extracellular, transmembrane and intracellular  
region of the MCK-10 receptor. The regions of  
homology between CCK-2 and MCK-10 extend into the  
N-terminus consensus sequence for the discoidin I like  
15 family of proteins. (Poole et al. 1981, J. Mol. Biol.  
153, 273-289). The homology between CCK-2 and MCK-10  
is diagramed in Figure 4A and 4B.

### 8. DEPOSIT OF MICROORGANISMS

20 The following organisms were deposited with the  
American Type Culture Collection (ATCC), 12301  
Parklawn Drive, Rockville, Maryland 20852.

	<u>Strain Designation</u>	<u>Containing</u>	<u>Accession No.</u>
25	CCK-2	pCCK-2	69468
	MCK-10-1	pMCK-10-1	69464
	MCK-10-2	pMCK-10-2	69465
	MCK-10-3	pMCK-10-3	69466
	MCK-10-4	pMCK-10-4	69467

30 The present invention is not to be limited in  
scope by the exemplified embodiments or deposited  
organisms which are intended as illustrations of  
single aspects of the invention, and any clones, DNA  
35 or amino acid sequences which are functionally

5

**10**

35